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(54) Title: USE OF INDOLINONE COMPOUNDS AS MODULATORS OF PROTEIN KINASES

(57) Abstract

The present invention relates to organic molecules capable of modulating tyrosine kinase signal transduction in order to regulate, modulate and/or inhibit abnormal cell proliferation.

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USE OF INDOLINONE COMPOUNDS AS MODULATORS OF PROTEIN KINASES

DESCRIPTION

RELATED APPLICATIONS

This application relates to U.S. patent application Serial No. 60/031,587, filed December 5, 1996, entitled "Tyrosine, Serine, and Threonine Kinase Modulators and Related Products and Methods for the Treatment of Disease" by McMahon et al. (Lyon & Lyon Docket No. 223/058) and this application also relates to U.S. patent application Serial No. 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products and Methods for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187) which is a continuation-in-part application of U.S. patent applications Serial Nos. 08/655,225, filed June 5, 1996, entitled "3-(2'Halobenzylidenyl)-2-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/299); 08/655,226, filed June 5, 1996, entitled "3-(4'-Dimethylaminobenzylidenyl)-2-Indolinone and Analogues Thereof for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/300); 08/655,223, filed June 5, 1996, entitled "3-Heteroaryl-2-Indolinone Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/301); 08/655,224, filed June 5, 1996, entitled "3-

(2'-Alkoxybenzylidenyl)-2-Indolinone and Analogues Thereof for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/302); and, 08/659,191, filed June 5, 1996, entitled "3-(4'Bromobenzylindenyl)-2-Indolinone and Analogues Thereof for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/303), all of which are continuations-in-part of U.S. patent application Serial No. 08/485,323, filed June 7, 1995, entitled "Benzylidene-Z-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/298) all of which are incorporated herein by reference in their entirety, including any drawings.

INTRODUCTION

The present invention relates to novel compounds capable of modulating, regulating and/or inhibiting tyrosine serine, and/or kinase signal transduction. The present invention is also directed to methods of regulating, modulating or inhibiting tyrosine theonine kinases, whether of the receptor or non-receptor class, for the prevention and/or treatment of disorders related to unregulated tyrosine kinase signal transduction, including cell proliferative and metabolic disorders.

BACKGROUND OF THE INVENTION

The following description of the background of the invention is provided to aid in understanding the invention, but is not admitted to be or describe prior art to the invention.

Protein kinases and protein phosphatases regulate a wide variety of cellular processes including metabolism cell proliferation, cell differentiation, and cell survival by participating in signal transduction pathways. Alterations in the cellular function of a protein kinase or protein phosphatase can give rise to various diseased states in an organism. For example, many types of cancer tumors are associated with increases in the activity of specific protein kinases. Cell and tissue degeneration can also be associated with decreases in the activity of particular protein kinases.

Cellular signal transduction is a fundamental mechanism whereby extracellular stimuli are relayed to the interior of cells. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of proteins. Phosphorylation of amino acids regulates the activity of mature proteins by altering their structure and function.

Phosphate most often resides on the hydroxyl moiety of serine, threonine, or tyrosine amino acids in proteins. Enzymes that mediate phosphorylation of cellular effectors fall into two classes. While protein phosphatases hydrolyze phosphate moieties from phosphoryl protein substrates, protein kinases transfer a phosphate moiety from adenosine triphosphate to protein substrates. The converse functions of protein kinases and protein phosphatases balance and regulate the flow of signals in signal transduction processes.

Protein kinases are divided into two groups - receptor and non-receptor type proteins. Receptor protein kinases comprise an extracellular region, a transmembrane region, and an intracellular region. Part of the intracellular region of receptor protein kinases harbors a catalytic domain. While non-receptor protein kinases do not harbor extracellular or transmembrane regions, they do comprise a region similar to the intracellular regions of their receptor counterparts.

Protein kinases are divided further into three classes based upon the amino acids they act upon. Some incorporate phosphate on serine or threonine only, some incorporate phosphate on tyrosine only, and some incorporate phosphate on serine, threonine, and tyrosine.

In an effort to discover novel treatments for diseases, biomedical researchers and chemists have designed, synthesized, and tested molecules that inhibit the function of protein kinases. Some small organic molecules form a class of compounds that modulate the function of protein kinases. Examples of molecules that have been reported to inhibit the function of protein kinases are bis monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO 92/20642, published November 26, 1992 by Maguire et al.), vinylene-azaindole derivatives (PCT WO 94/14808, published July 7, 1994 by Ballinari et al.), 1-cyclopropyl-4-pyridyl-quinolones (U.S. Patent No. 5,330,992), styryl compounds (U.S. Patent No.

5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427, published February 17, 1994 by Denny et al.), tricyclic polyhydroxylic compounds (PCT WO 92/21660, published December 10, 1992 by Dow), and benzylphosphonic acid compounds (PCT WO 91/15495, published October 17, 1991 by Dow et al). The compounds that can traverse cell membranes and are resistant to acid hydrolysis are potentially advantageous therapeutics as they can become highly bioavailable after being administered orally to patients. However, many of these protein kinase inhibitors only weakly inhibit the function of protein kinases. In addition, many inhibit a variety of protein kinases and will therefore cause multiple side-effects as therapeutics for diseases.

Some indolinone compounds, however, form classes of acid resistant and membrane permeable organic molecules. WO 96/22976, published August 1, 1996 by Ballinari et al., describes hydrosoluble indolinone compounds that harbor tetralin, naphthalene, quinoline, and indole substituents fused to the oxindol ring. These bicyclic substituents are in turn substituted with polar moieties including hydroxylated alkyl, phosphate, and ether moieties. U.S. Patent Application Serial Nos. 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products an Methods for the Treatment of Disease" by Tang et al. (Lyon &

Lyon Docket No. 221/187) and 08/485,323, filed June 7, 1995, entitled "Benzylidene-Z-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/298) and International Patent Publication WO 96/22976, published August 1, 1996 by Ballinari et al., all of which are incorporated herein by reference in their entirety, including any drawings, describe indolinone chemical libraries of indolinone compounds harboring other bicyclic moieties as well as monocyclic moieties fused to the oxindol ring. Applications 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products an Methods for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187); 08/485,323, filed June 7, 1995, entitled "Benzylidene-Z-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/298), and WO 96/22976, published August 1, 1996 by Ballinari et al., teach methods of indolinone synthesis, methods of testing the biological activity of indolinone compounds in cells, and inhibition patterns of indolinone derivatives.

Despite the significant progress that has been made in developing indolinone based pharmaceuticals, there remains a need in the art to identify the particular structures and substitution patterns that cause inhibition of particular protein kinases and other specified biological activities.

SUMMARY OF THE INVENTION

The present invention relates to organic molecules capable of modulating, regulating and/or inhibiting tyrosine, serine, and/or threonine kinase signal transduction. Such compounds are useful for the treatment of diseases related to unregulated signal transduction, including cell proliferative diseases such as cancer, atherosclerosis, arthritis and restenosis and metabolic diseases such as diabetes.

The present invention features indolinone compounds that potently inhibit protein kinases. The data provided herein demonstrates that the compounds of the invention can inhibit tyrosine kinases, serine kinases, and threonine kinases. Given this information, those skilled in the art will be able to target particular disease indications [see Section III - Target Diseases below] based upon the identification of the particular compound either as a tyrosine serine or theronine kinase inhibitor. The invention also features methods of synthesizing the compounds, pharmaceutical compositions comprising the compounds, and methods of preventing and treating abnormal conditions in an organism.

The compounds of the invention represent a new generation of potential cancer therapeutics as they are specific to their target and will subsequently cause few side effects. These properties are welcome improvements over the currently utilized cancer therapeutics that cause multiple side effects and deleteriously weaken patients.

Thus, in a first aspect, the invention features a method for modulating the activity of a protein kinase. The method involves contacting said kinase with an indolinone compound or pharmaceutically acceptable salt thereof demonstrated to have modulating capability in a bioassay corresponding to the kinase being modulated. The protein kinase may be a serine kinase, a threonine kinase, or a tyrosine kinase and may be either a split kinase or a non-split kinase. Typical bioassays are listed in Table 1 and include Flk, EGF, HER2, PCRB, BIOKIN, and rafmak assays. Such assays are known by those skilled in the art to correlate with tyrosine kinase activity, serine kinase activity or threonine kinase activity depending upon which particular is used.

The term "indolinone" is used as that term is commonly understood in the art and includes a large subclass of substituted or unsubstituted compounds that are capable of being synthesized from an aldehyde moiety and an oxindol moiety. Indolinones are a large class of molecules as nearly any aldehyde and nearly any oxindol may be utilized in the reaction.

The term "pharmaceutically acceptable salt" refers to a formulation of a compound that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the compound. Pharmaceutical salts can be obtained by reacting a compound of the invention with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid,

methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid and the like.

In another aspect, the invention features an indolinone compound that modulates the catalytic activity of a protein kinase. Examples of such compounds are listed in Table 1.

The term "modulate" refers to a compound that alters the catalytic activity of a protein kinase. A modulator preferably activates the catalytic activity of a protein kinase, more preferably activates or inhibits the catalytic activity of a protein kinase depending on the concentration of the compound exposed to the protein kinase, or most preferably inhibits the catalytic activity of a protein kinase.

The term "protein kinase" defines a class of proteins that regulate a variety of cellular functions. Protein kinases regulate cellular functions by reversibly phosphorylating protein substrates which thereby changes the conformation of the substrate protein. The conformational change modulates catalytic activity of the substrate or its ability to interact with other binding partners.

The term "catalytic activity", in the context of the invention, defines the rate at which a protein kinase phosphorylates a substrate. Catalytic activity can be measured, for example, by determining the amount of a substrate converted to a product as a function of time. Phosphorylation of a substrate occurs at the active-site of a protein kinase. The active-site is

normally a cavity in which the substrate binds to the protein kinase and is phosphorylated.

Inhibitors of protein kinase catalytic activity are known in the art. Small molecule inhibitors typically block the binding of substrates by tightly interacting with the protein kinase active-site. Indolinone compounds, for example, can bind to the active-site of a protein kinase and inhibit the molecule effectively, as measured by inhibition constants on the order of 10^{-6} M.

A preferred embodiment of the invention relates to an indolinone compound that inhibits the catalytic activity of a *FLK* protein kinase. The indolinone preferably inhibits the catalytic activity of the *FLK* protein kinase with an IC_{50} less than 50 μM, more preferably with an IC_{50} less than 5 μM, and most preferably with an IC_{50} less than 0.5 μM.

Another preferred embodiment of the invention relates to an indolinone compound that inhibits the catalytic activity of a platelet derived growth factor protein kinase. The indolinone preferably inhibits the catalytic activity of the platelet derived growth factor protein kinase with an IC_{50} less than 50 μM, more preferably with an IC_{50} less than 5 μM, and most preferably with an IC_{50} less than 0.5 μM.

The term "*FLK*" refers to a protein kinase that phosphorylates protein substrates on tyrosine residues. The *FLK* protein kinase regulates cellular functions in response to the VEGF growth factor. These cellular functions include, but are not limited to, cellular

proliferation, and in particular, blood vessel proliferation in tissues.

The term "platelet derived growth factor" refers to a protein kinase that phosphorylates substrates on tyrosine residues. The platelet derived growth factor protein kinase regulates cellular functions in response to the PDGF growth factor. These cellular functions include, but are not limited to, cellular proliferation.

The term "IC_{50}", in the context of the invention, refers to a parameter that describes the concentration of a particular indolinone required to inhibit 50% of the *FLK* protein kinase or platelet derived growth factor protein kinase catalytic activity. The IC50 parameter can be measured using an assay described herein and by varying the concentration of a particular indolinone compound.

Another aspect of the invention features a pharmaceutical composition comprising an oxidolinone compound of the invention and a physiologically acceptable carrier or diluent.

The term "pharmaceutical composition" refers to a mixture of an indolinone compound of the invention with other chemical components, such as diluents or carriers. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds

with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid and the like.

The term "physiologically acceptable" defines a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the compound.

The term "carrier" defines a chemical compound that facilitates the incorporation of a compound into cells or tissues. For example dimethyl sulfoxide (DMSO) is a commonly utilized carrier as it facilitates the uptake of many organic compounds into the cells or tissues of an organism.

The term "diluent" defines chemical compounds diluted in water that will dissolve the compound of interest as well as stabilize the biologically active form of the compound. Salts dissolved in buffered solutions are utilized as diluents in the art. One commonly used buffered solution is phosphate buffered saline because it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a compound.

A final aspect of the invention features a method of preventing or treating an abnormal condition in an organism. The abnormal condition is associated with an aberration in a signal transduction pathway characterized by an interaction between a protein kinase

and a natural binding partner. The method comprises the following steps: (a) administering a compound of the invention to an organism; and (b) promoting or disrupting the abnormal interaction.

The term "preventing" refers to a method of barring the organism from acquiring the abnormal condition.

The term "treating" refers to a method of alleviating or abrogating the abnormal condition in the organism.

The term "organism" relates to any living entity comprised of at least one cell. An organism can be as simple as one eukaryotic cell or as complex as a mammal.

The term "abnormal condition" refers to a function in the cells or tissues of an organism that deviates from their normal functions in that organism. An abnormal condition can relate to cell proliferation, cell differentiation, or cell survival.

Aberrant cell proliferative conditions include cancers such as fibrotic and mesangial disorders, abnormal angiogenesis and vasculogenesis, wound healing, psoriasis, diabetes mellitus, and inflammation.

Aberrant differentiation conditions include, but are not limited to neurodegenerative disorders, slow wound healing rates, and tissue grafting techniques.

Aberrant cell survival conditions relate to conditions in which programmed cell death (apoptosis) pathways are activated or abrogated. A number of protein kinases are associated with the apoptosis pathways. Aberrations in the function of any one of the

protein kinases could lead to cell immortality or premature cell death.

Cell proliferation, differentiation, and survival are phenomena simply measured by methods in the art. These methods can involve observing the number of cells or the appearance of cells under a microscope with respect to time (days).

The term "administering" relates to a method of incorporating a compound into cells or tissues of an organism. The abnormal condition can be prevented or treated when the cells or tissues of the organism exist within the organism or outside of the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. For cells harbored within the organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, injection, and aerosol applications. For cells outside of the organism, multiple techniques exist in the art to administer the compounds, including (but not limited to) cell microinjection techniques, transformation techniques, and carrier techniques.

The aberrant condition can also be prevented or treated by administering a group of cells having an aberration in a signal transduction process to an organism. The effect of administering a compound on organism function can then be monitored. The art contains multiple methods of introducing a group of cells to an organism as well as methods of administering a compound to an organism. The organism is preferably a

frog, more preferably a mouse, rat, rabbit, guinea pig, or goat, and most preferrably a monkey or ape.

The term "signal transduction pathway" refers to the molecules that propagate an extracellular signal through the cell membrane to become an intracellular signal. This signal can then stimulate a cellular response. The polypeptide molecules involved in signal transduction processes are typically receptor and non-receptor protein kinases, receptor and non-receptor protein phosphatases, nucleotide exchange factors, and transcription factors.

The term "aberration", in conjunction with a signal transduction process, refers to a protein kinase that is over- or under-expressed in an organism, mutated such that its catalytic activity is lower or higher than wild-type protein kinase activity, mutated such that it can no longer interact with a natural binding partner, is no longer modified by another protein kinase or protein phosphatase, or no longer interacts with a natural binding partner.

The term "natural binding partner" refers to a polypeptide that normally binds to the intracellular region of a protein kinase in a cell. These natural binding partners can play a role in propagating a signal in a protein kinase signal transduction process. The natural binding partner can bind to a protein kinase intracellular region with high affinity. High affinity represents an equilibrium binding constant on the order of 10^{-6} M or less. However, a natural binding partner

can also transiently interact with a protein kinase intracellular region and chemically modify it. Protein kinase natural binding partners are chosen from a group consisting of, but not limited to, *src* homology 2 (SH2) or 3 (SH3) domains, other phosphoryl tyrosine binding (PTB) domains, and other protein kinases or protein phosphatases.

The term "promoting or disrupting the abnormal interaction" refers to a method that can be accomplished by administering a compound of the invention to cells or tissues in an organism. A compound can promote an interaction between a protein kinase and natural binding partners by forming favorable interactions with multiple amino acids at the complex interface. Alternatively, a compound can inhibit an interaction between a protein kinase and natural binding partners by compromising favorable interactions formed between amino acids at the complex interface.

A preferred embodiment of the invention relates to the method of treating an abnormal condition in an organism, where the organism is a mammal.

The term "mammal" refers preferably to such organisms as mice, rats, rabbits, guinea pigs, and goats, more preferably to monkeys and apes, and most preferably to humans.

Another preferred embodiment of the invention relates to a method of treating or preventing an abnormal condition associated with the *FLK* protein kinase.

In yet anther preferred embodiment, the invention relates to a method of treating or preventing an abnormal condition associated with the platelet derived growth factor protein kinase.

BRIEF DESCRIPTION OF THE DRAWINGS

Table 1 shows illustrative compounds of the invention and the corresponding bioassay which has been used to identify the compound as a tyrosine kinase modulator, serine kinase modulator and/or threonine kinase modulator.

In Table 1, certain abbreviations are used to refer to particular assays as follows:

cprb, cprb-5 - cellular (i.e., whole cells) PDGFR assay - Example 2(a)(iii)

celegf - cellular EGFR assay - Example 2(a)(v)

mflk2 - cellular Flk assay - Example 2(a)(i)

her2 - cellular Her2 assay - Example 2(a)(ii)

celigf1 - cellular IGF1 assay - Example 2(a)(iv)

biokin - biochemical EGFR assay - Example 2(a)(vii)

rafmek - biochemical (i.e., lysed cells) Raf assay - Example 2(a)(vi)

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed in part towards designing compounds that modulate the function of protein kinases. Indolinone compounds that are modified with substituents in the manner set forth herein, effectively modulate the

function of protein kinases. These modulating compounds can therefore be directed towards preventing or treating abnormal conditions in organisms associated with over-active protein kinase function by specifically inhibiting the function of the protein kinase. Alternatively, the modulating compounds can be directed towards preventing or treating abnormal conditions in organisms associated with under-active or non-active protein kinase function by specifically activating the function of the protein kinase.

I. Synthesis of Indolinone Compounds

The indolinone compounds of the invention are synthesized by reacting an aldehyde with an oxindol. Examples of indolinone compounds of the invention are depicted in Table 1. Descriptions of methods for synthesizing indolinone compounds are described in U.S. Application Serial No. 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products an Methods for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187) (incorporated herein by reference in its entirety, including any drawings) and in the examples described herein. The examples fully describe the solvents, temperatures, separation techniques, and other conditions utilized for the invention. Other methods that may be used or modified by those skilled in the art are described in International Patent Publication number WO 96/22976, published August 1, 1996 by Ballinari et

al., incorporated herein by reference in its entirety, including any drawings. Descriptions of the methods used to specifically synthesize the indolinone compounds of the invention, as shown in Table 1, are disclosed herein.

II. Biological Activity of Indolinone Compounds

Indolinone compounds of the invention can be tested for their ability to activate or inhibit protein kinases in biological assays. The methods used to measure indolinone modulation of protein kinase function are described in U.S. Application Serial No. 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products an Methods for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187) incorporated herein by reference in its entirety, including any drawings. Indolinone compounds of the invention were tested for their ability to inhibit the various protein kinases. The biological assay and results of these inhibition studies are reported herein.

III. Target Diseases to be Treated by Indolinone Compounds

Protein kinases are essential regulatory molecules that control a variety of cellular functions. For this reason, any alteration in the function of a protein kinase can cause an abnormal condition in an organism.

One of the many functions controlled by protein kinases is cell proliferation.

Alterations in the function of a protein kinase that normally regulates cell proliferation can lead to enhanced or decreased cell proliferative conditions evident in certain diseases. Aberrant cell proliferative conditions include cancers such as fibrotic and mesangial disorders, abnormal angiogenesis and vasculogenesis, wound healing, psoriasis, restenosis, diabetes mellitus, and inflammation.

Fibrotic disorders relate to the abnormal formation of the cellular extracellular matrix. An example of a fibrotic disorder is hepatic cirrhosis. Hepatic cirrohis is characterized by an increased concentration of extracellular matrix constituents resulting in the formation of a hepatic scar. Hepatic cirrhosis can cause diseases such as cirrhosis of the liver.

Mesangial cell proliferative disorders occur due to the abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies.

Angiogenic and vasculogenic disorders result from excess proliferation of blood vessels. Blood vessel proliferation is necessary in a variety of normal physiological processes such as embryonic development, corpus luteum formation, wound healing and organ

regeneration. However, blood vessel proliferation is also essential in cancer tumor development. Other examples of blood vessel proliferative disorders include arthritis, where new capillary blood vessels invade the joint and destroy cartilage. In addition, blood vessel proliferative diseases include ocular diseases, such as diabetic retinopathy, where new capillaries in the retina invade the vitreous, bleed and cause blindness. Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated in adverse regulation of RPKs or RPPs.

Angiogenic and vasculogenic disorders are closely linked to the *FLK* protein kinase. *FLK*-1 is activated upon binding VEGF, a strong regulator for endothelial cell proliferation as well as normal and pathological angiogenesis. Klagsburn and Soker, 1993, *Current Biology* 3:699-702. Thus, compounds that specifically inhibit the *FLK* protein kinase are potential anti-cancer agents as they may decrease the vasculature that nourishes tumors. These inhibitors will most likely result in minimizing and even obliterating solid tumors. In addition, compounds that specifically inhibit *FLK* will potentially represent a new generation of cancer therapeutics as they will most likely cause few side effects. These potential properties are a significant improvement over the currently utilized cancer therapeutics that cause multiple side effects and deleteriously weaken patients.

In addition to cell proliferation, some RPKs and RPPs regulate the pentultimate cellular functions, cell survival and cell death. Glial derived growth factor (GDNF) activates *c-ret*, for example, by bringing multiple *c-ret* receptors together into close proximity and promoting cross phosphorylation of the intracellular regions. Signal transduction molecules that form a complex with *c-ret* as a result of these phosphoryl moieties, such as *grb-2*, *sos*, *ras*, and *raf*, propagate a signal in the cell that promotes neural survival. Thus, compounds that promote the interactions of these stimulatory molecules of *c-ret* would enhance the activity of *c-ret*. Alternatively, protein phosphatases can remove the phosphoryl moieties placed on the intracellular region of *c-ret* in response to GDNF, and thus inhibit the signaling capability of *c-ret*. Thus compounds that inhibit phosphatases of *c-ret* will enhance the signaling capacity of *c-ret*. In the context of the present invention, the *c-ret* protein kinase could be activated by particular indolinone compounds.

c-ret is implicated in the development and survival of enteric, synaptic, and sensory neurons and neurons of the renal system upon stimulation by GDNF. Lack of function mutations in *c-ret* can lead to Hirschsprung's disease, for example, which manifests itself as a decrease in intestinal tract innervation in patients. Thus, compounds that activate *c-ret* are potential therapeutic agents for the treatment of neurodegenerative disorders, including, but not limited

to, Hirschsprung's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. Compounds that inhibit *c-ret* function are possible anti-cancer agents as over-expression of *ret* in cells is implicated in cancers, such as cancer of the thyroid.

PDGFR kinase inhibitors can be used to treat cancer (glioma et al), arterial restenosis, fibrotic diseases of the lung, kidney and liver, would scarring.

Flk kinase inhibitors can be used to treat endothelial proliferation and diseases including cancer, metastatic disease, rheumatoid arthritis, psoriasis, and ocular diseases including diabetic retinopaties and age-related macular degeneration.

EGFR and HER2 kinase inhibitors can be sued to treat cancer (breast, ovarian, squamous cell carcinoma, et al tumors), psoriasis (keritinocyte proliferation).

Raf kinase inhibitors can be used to treat cancer, hyperplasia in arterial restenosis, transplant rejection, inflammation, psoriasis, etc.

IV. Pharmaceutical Compositions and Administration of Indolinone Compounds

Methods of preparing pharmaceutical formulations of the compounds, methods of determining the amounts of compounds to be administered to a patient, and modes of administering compounds to an organism are disclosed U.S. Application Serial No. 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products an Methods for the Treatment of

Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187) incorporated herein by reference in its entirety, including any drawings. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

EXAMPLES

The examples below are not limiting and are merely representative of various aspects and features of the present invention. The examples demonstrate methods of synthesizing indolinone compounds of the invention. The examples also demonstrate the specificity as well as the potency with which these compounds inhibit protein kinase function in cells.

EXAMPLE 1: Synthesis of Indolinone Compounds of the Invention

The compounds of the present invention may be synthesized according to known techniques. The following represent preferred methods for synthesizing the compounds of the claimed invention.

(a) General Syntheses of Indolinone Analogs

The following general methodologies are used to synthesize 3-substituted-2-indolinone compounds of the invention.

(i) M thod A

A reaction mixture of the proper oxindole (2-indolinone) (1 equiv.), the appropriate aldehyde (1.2 equiv.), and piperidine (0.1 equiv.) in ethanol (1 - 2 mL / 1 mmol oxindole) is stirred at 90°C for 3 - 5 h. After cooling, the precipitate is filtered, washed with cold ethanol, and dried to yield the target compound.

(ii) Method B

To each well of 96-well reaction plate was dispensed 100μL of 0.25 M oxindole in DMSO, 200μL of 0.125 M aldehyde in ethanol, 50μL of 0.05 M piperidine in ethanol, and 25μL of 0.05 M of acetic acid in ethanol. The reaction blocks were then heated to 80°C for 24 hr. Ethanol was then removed under vacuum. The resulting DMSO solution of the reaction residue was then frozen.

EXAMPLE 2: *In Vitro* RTK Assays

The following *in vitro* assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the RTKs. Similar assays can be designed along the same lines for any tyrosine kinase using techniques well known in the art.

(a) Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) may be used to detect and measure the presence of tyrosine kinase activity. The ELISA may be conducted according

to known protocols which are described in, for example, Voller, *et al.*, 1980, "Enzyme-Linked Immunosorbent Assay," In: *Manual of Clinical Immunology, 2d ed.*, edited by Rose and Friedman, pp 359-371 Am. Soc. Of Microbiology, Washington, D.C.

The disclosed protocol may be adapted for determining activity with respect to a specific RTK. For example, the preferred protocols for conducting the ELISA experiments for specific RTKs is provided below. Adaptation of these protocols for determining a compound's activity for other members of the RTK family, as well as non-receptor tyrosine kinases, are within the scope of those in the art.

(i) FLK-1 ELISA

An ELISA assay was conducted to measure the kinase activity of the FLK-1 receptor and more specifically, the inhibition or activation of protein tyrosine kinase activity on the FLK-1 receptor. Specifically, the following assay was conducted to measure kinase activity of the FLK-1 receptor in FLK-1/NIH3T3 cells.

Materials And Methods.

Materials. The following reagents and supplies were used:

a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);

b. Cappel goat anti-rabbit IgG (catalog no. 55641);

c. PBS (Gibco Catalog No. 450-1300EB);

d. TBSW Buffer (50 mM Tris (pH 7.2), 150 mM NaCl and 0.1% Tween-20);

e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4°C);

f. HNTG buffer (20mM HEPES buffer (pH 7.5), 150mM NaCl, 0.2% Triton X-100, and 10% glycerol);

g. EDTA (0.5 M (pH 7.0) as a 100X stock);

h. Sodium ortho vanadate (0.5 M as a 100X stock);

i. Sodium pyro phosphate (0.2M as a 100X stock);

j. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);

k. NIH3T3 C7#3 Cells (FLK-1 expressing cells);

l. DMEM with 1X high glucose L Glutamine (catalog No. 11965-050);

m. FBS, Gibco (catalog no. 16000-028);

n. L-glutamine, Gibco (catalog no. 25030-016);

o. VEGF, PeproTech, Inc. (catalog no. 100-20) (kept as 1 μg/100 μl stock in Milli-Q dH_2O and stored at -20°C;

p. Affinity purified anti-FLK-1 antiserum;

q. UB40 monoclonal antibody specific for phosphotyrosine (*see*, Fendley, *et al.*, 1990, *Cancer Research* 50:1550-1558);

r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011);

s. 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) solution (100mM citric

acid (anhydrous), 250 mM Na_2HPO_4 (pH 4.0), 0.5 mg/ml ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at 4°C until ready for use;

t. H_2O_2 (30% solution)(Fisher catalog no. H325);

u. ABTS/H_2O_2 (15ml ABTS solution, 2 μl H_2O_2) prepared 5 minutes before use and left at room temperature;

v. 0.2 M HCl stock in H_2O;

w. dimethylsulfoxide (100%)(Sigma Catalog No. D-8418); and

x. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049).

Protocol. The following protocol was used for conducting the assay:

1. Coat Corning 96-well elisa plates with 1.0μg per well Cappel Anti-rabbit IgG antibody in 0.1M Na_2C0_3 pH 9.6. Bring final volume to 150 μl per well. Coat plates overnight at 4°C. Plates can be kept up to two weeks when stored at 4°C.

2. Grow cells in Growth media(DMEM, supplemental with 2.0mM L-Glutamine, 10% FBS) in suitable culture dishes until confluent at 37°C, 5% CO_2.

3. Harvest cells by trypsinization and seed in Corning 25850 polystyrene 96-well roundbottom cell plates, 25.000 cells/well in 200μl of growth media.

4. Grow cells at least one day at 37°C, 5% CO_2.

5. Wash cells with D-PBS 1X.

6. Add 200μl/well of starvation media (DMEM, 2.0mM l-Glutamine, 0.1% FBS). Incubate overnight at 37°C, 5% CO_2.

7. Dilute Compounds/Extracts 1:20 in polypropylene 96 well plates using starvation media. Dilute dimethylsulfoxide 1:20 for use in control wells.

8. Remove starvation media from 96 well cell culture plates and add 162 μl of fresh starvation media to each well.

9. Add 18μl of 1:20 diluted Compound/Extract dilution (from step 7) to each well plus the 1:20 dimethylsulfoxide dilution to the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethylsulfoxide is 0.5 %. Incubate the plate at 37°C, 5% CO_2 for two hours.

10. Remove unbound antibody from ELISA plates by inverting plate to remove liquid. Wash 3 times with TBSW + 0.5% ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.

11. Block plates with TBSW + 0.5% Ethanolamine, pH 7.0, 150 μl per well. Incubate plate thirty minutes while shaking on a microtiter plate shaker.

12. Wash plate 3 times as described in step 10.

13. Add 0.5μg/well affinity purified anti-FLU-1 polyclonal rabbit antiserum. Bring final volume to 150μl/well with TBSW + 0.5% ethanolamine pH 7.0. Incubate plate for thirty minutes while shaking.

14. Add 180 μl starvation medium to the cells and stimulate cells with 20μl/well 10.0mM sodium ortho

vanadate and 500 ng/ml VEGF (resulting in a final concentration of 1.0mM sodium ortho vanadate and 50ng/ml VEGF per well) for eight minutes at 37°C, 5% CO_2. Negative control wells receive only starvation medium.

15. After eight minutes, media should be removed from the cells and washed one time with 200µl/well PBS.

16. Lyse cells in 150µl/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium ortho vanadate, sodium pyro phosphate and EDTA.

17. Wash ELISA plate three times as described in step 10.

18. Transfer cell lysates from the cell plate to elisa plate and incubate while shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.

20. Incubate ELISA plate with 0.02µg/well UB40 in TBSW + 05% ethanolamine. Bring final volume to 150µl/well. Incubate while shaking for 30 minutes.

21. Wash plate three times as described in step 10.

22. Incubate ELISA plate with 1:10,000 diluted EIA grade goat anti-mouse IgG conjugated horseradish peroxidase in TBSW + 0.5% ethanolamine, pH 7.0. Bring final volume to 150µl/well. Incubate while shaking for thirty minutes.

23. Wash plate as described in step 10.

24. Add 100 μl of ABTS/H_2O_2 solution to well. Incubate ten minutes while shaking.

25. Add 100 μl of 0.2 M HCl for 0.1 M HCl final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader at 410 nm.

(ii) HER-2 ELISA

Assay 1: EGF Receptor-HER2 Chimeric Receptor Assay In Whole Cells. HER2 kinase activity in whole EGFR-NIH3T3 cells was measured as described below:

Materials and Reagents. The following materials and reagents were used to conduct the assay:

a. EGF: stock concentration= 16.5 ILM; EGF 201, TOYOBO, Co., Ltd. Japan.

b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

c. Anti-phosphotyrosine antibody (anti-Ptyr) (polyclonal)(*see*, Fendley, *et al.*, *supra*).

d. Detection antibody: Goat anti-rabbit lgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.

e. TBST buffer:

Tris-HCl, pH 7.2	50 mM
NaCl	150 mM
Triton X-100	0.1

f. HNTG 5X stock:

HEPES	0.1 M
NaCl	0.75 M
Glycerol	50%

Triton X-100	1.0%

g. ABTS stock:

Citric Acid	100 mM
Na_2HPO_4	250 mM
HCl, conc.	0.5 pM
ABTS*	0.5mg/ml

* (2,2' -azinobis(3-ethylbenzthiazolinesulfonic acid)). Keep solution in dark at 4°C until use.

h. Stock reagents of:

EDTA 100 mM pH 7.0
Na_3VO_4 0.5 M
$Na_4(P_2O_7)$ 0.2 M

Procedure. The following protocol was used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody at 0.5 g per well in PBS, 100 μl final volume/well, and store overnight at 4°C. Coated plates are good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and replace with 100 μl blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to use, remove blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. An NIH3T3 cell line overexpressing a chimeric receptor containing the EGFR extracellular

domain and extracellular HER2 kinase domain can be used for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% fetal bovine serum. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1500 rpm, at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum) , and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37°C for about 40 hours.

C. Assay Procedures

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 l to a TBST well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO_2 at 37°C for two hours.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μl dilute EGF (1:12 dilution), 100 nM final concentration is attained.

3. Prepare fresh HNTG* sufficient for 100 μl per well; and place on ice.

HNTG* (10 ml):

HNTG stock	2.0 ml
milli-Q H_2O	7.3 ml

EDTA, 100 mM, pH 7.0	0.5 ml
Na_3VO_4, 0.5 M	0.1 ml
$Na_4(P_2O_7)$, 0.2 M	0.1 ml

4. After 120 minutes incubation with drug, add prepared SGF ligand to cells, 10 μl per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

5. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μl per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

6. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

7. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

8. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO anti-rabbit IgG antibody to the ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30

minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

9. Remove TAGO detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 μl per well. Incubate shaking at room temperature for 20 minutes. (ABTS/H_2O_2 solution: 1.0 μl 30% H_2O_2 in 10 ml ABTS stock).

10. Stop reaction by adding 50 μl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

11. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

Assay 2: HER-2-BT474 ELISA. A second assay may be conducted to measure whole cell HER2 activity. Such assay may be conducted as follows:

Materials And Reagents. The following materials and reagents were used:

a. BT-474 (ATCC HBT20), a human breast tumor cell line which expresses high levels of HER2 kinase.

b. Growth media comprising RPMI + 10% FBS + GMS-G (Gibco supplement) + glutamine for use in growing BT-474 in an incubator with 5% CO_2 at 37°C.

c. A monoclonal anti-HER2 antibody.

d. D-PBS:

KH_2HPO_4	0.20 g/l	10 (GIBCO,310-4190AJ)
K_2HPO_4	2.16 g/l	
KCl	0.20 g/l	
NaCl	8.00 g/l	(pH 7.2)

e. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).

f. TBST buffer:

Tris-HCl	50 mM
NaCl	150 mM (pH 7.2, HCl 10 N)
Triton X-100	0.1%

wherein stock solution of TES (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

g. HNTG buffer (5x):

HEPES	0.1 M
NaCl	750 mM (pH 7.2 (HCl, 1 N)
Glycerol	50%
Triton X-100	1.0%

Stock solution (5x) is prepared and kept in 4°C.

h. EDTA-HCl: 0.5 M pH 7.0 (10 N HCl) as 500X stock.

i. Na_3VO_4: 0.5 M as 100X stock is kept at -80°C as aliquots.

j. $Na_4(P_2O_7)$: 0.2 M as 100X stock.

k. Polyclonal antiserum anti-phosphotyrosine.

l. Goat anti-rabbit IgG, horseradish peroxidase (POD) conjugate (detection antibody), Tago (Cat. No. 4520; Lot No. 1802): Tago, Inc., Burlingame, CA.

m. ABTS solution:

Citric acid	100 mM
Na_2HPO_4	250 mM (pH 4.0, 1 N HCl)
ABTS	0.5 mg/ml

wherein ABTS is 2.2'-azinobis(3-ethylbenzthiazoline sulfonic acid). For this assay, the ABTS solution should be kept in the dark at 4°C. The solution should be discarded when it turns green.

n. Hydrogen peroxide: 30% solution is kept in dark and 4°C.

Procedure. All the following steps are at room temperature and aseptically, unless stated otherwise. All ELISA plate washing is by rinsing with distilled water three times and once with TBST.

A. Cell Seeding

1. Grow BT474 cells in tissue culture dishes (Corning 25020-100) to 80-90% confluence and collect using Trypsin-EDTA (0.25%, GIBCO).

2. Resuspend the cells in fresh medium and transfer to 96-well tissue culture plates (Corning, 25806-96) at about 25,000-50,000 cells/well (100 μl/well) . Incubate the cells in 5% CO_2 at 37°C overnight.

B. ELISA Plate Coating and Blocking

1. Coat the ELISA plate (Corning 25805-96) with anti HER2 antibody at 0.5 μg/well in 150 μl PBS overnight at 4°C, and seal with parafilm. The antibody coated plates can be used up to 2 weeks, when stored at 4°C.

2. On the day of use, remove the coating solution, replace with 200 μl of Blocking Buffer, shake

the plate, and then remove the blocking buffer and wash the plate just before adding lysate.

C. Assay Procedures

1. TBST the drugs in serum-free condition. Before adding drugs, the old media is replaced with serum-free RPMI (90 μl/well)

2. Dilute drug stock (in 100% DMSO) 1:10 with RPMI, and transfer 10 μl/well of this solution to the cells to achieve a final drug DMSO concentration at 1%. Incubate the cells in 5% CO_2 at 37°C.

3. Prepare fresh cell lysis buffer (HNTG*)

5xHNTG	2 ml
EDTA	0.2 ml
Na_3VO_4	0.1 ml
$Na_4P_2O_7$	0. 1 ml
H_20	7.3 ml

4. After drug preincubation for two hours remove all the solution from the plate, transfer HNTG* (100 μl/well) to the cells, and shake for 10 minutes.

5. Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispensing. Transfer all the lysate to the ELISA plate and shake for 1 hour.

6. Remove the lysate, wash the plate, add anti-pTyr (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

7. Remove anti-pTyr, wash the plate, add goat anti-rabbit IgG conjugated antibody (1:5,000 with TBST) 100 μl/well, and shake for 30 minutes.

8. Remove anti-rabbit IgG antibody, wash the plate, and add fresh ABTS/H_2O_2 (1.2 μl H_2O_2 to 10 ml ABTS) 100 l/well to the plate to start color development, which usually takes 20 minutes.

9. Measure OD 410 nM, Dynatec MR5000.

(iii) PDGF-R ELISA

All cell culture media, glutamine, and fetal bovine serum were purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells were grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines were routinely subcultured twice a week and were negative for mycoplasma as determined by the Mycotect method (Gibco).

For ELISA assays, cells (U1242, obtained from Joseph Schlessinger, NYU) were grown to 80-90% confluency in growth medium (MEM with 10% FBS, NEAA, 1 mM NaPyr and 2 mM GLN) and seeded in 96-well tissue culture plates in 0.5% serum at 25,000 to 30,000 cells per well. After overnight incubation in 0.5% serum-containing medium, cells were changed to serum-free medium and treated with test compound for 2 hr in a 5% CO_2, 37°C incubator. Cells were then stimulated with ligand for 5-10 minute followed by lysis with HNTG (20 mM Hepes, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM Na_3VO_4, 0.2% Triton X-100, and 2 mM NaPyr). Cell lysates (0.5 mg/well in PBS) were transferred to ELISA plates previously coated with receptor-specific antibody and which had been blocked with 5% milk in TBST (50 mM Tris-

HCl pH 7.2, 150 mM NaCl and 0.1% Triton X-100) at room temperature for 30 min. Lysates were incubated with shaking for 1 hour at room temperature. The plates were washed with TBST four times and then incubated with polyclonal anti-phosphotyrosine antibody at room temperature for 30 minutes. Excess anti-phosphotyrosine antibody was removed by rinsing the plate with TBST four times. Goat anti-rabbit IgG antibody was added to the ELISA plate for 30 min at room temperature followed by rinsing with TBST four more times. ABTS (100 mM citric acid, 250 mM Na_2HPO_4 and 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) plus H_2O_2 (1.2 mL 30% H_2O_2 to 10 ml ABTS) was added to the ELISA plates to start color development. Absorbance at 410 nm with a reference wavelength of 630 nm was recorded about 15 to 30 min after ABTS addition.

(iv) IGF-I ELISA

The following protocol may be used to measure phosphotyrosine level on IGF-I receptor, which indicates IGF-I receptor tyrosine kinase activity.

Materials And Reagents. The following materials and reagents were used:

a. The cell line used in this assay is 3T3/IGF-1R, a cell line which overexpresses IGF-1 receptor.

b. NIH3T3/IGF-1R is grown in an incubator with 5% CO_2 at 37°C. The growth media is DMEM + 10% FBS (heat inactivated) + 2mM L-glutamine.

c. Anti-IGF-1R antibody named 17-69 is purified and used.

d. D-PBS:

KH_2PO_4	0.20 g/l
K_2HPO_4	2.16 g/l
KCl	0.20 g/l
NaCl	8.00 g/l (pH 7.2)

e. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).

f. TBST buffer:

Tris-HCl	50 mM
NaCl	150mM (pH 7.2/HCl 10N)
Triton X-100	0.1%

Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

g. HNTG buffer:

HEPES	20 mM
NaCl	150 mM (pH 7.2/HCl 1N)
Glycerol	10%
Triton X-100	0.2%

Stock solution (5X) is prepared and kept at 4°C.

h. EDTA/HCl: 0.5 M pH 7.0 (NaOH) as 100X stock.

i. Na_3VO_4: 0.5 M as 100X stock and aliquots are kept in -80°C.

j. $Na_4P_2O_7$: 0.2 M as 100X stock.

k. Insulin-like growth factor-1 from Promega (Cat# G5111).

l. Polyclonal antiserum anti-phosphotyrosine: rabbit sera or UB40 monoclonal antibody specific for phosphotyrosine.

m. Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520, Lot No. 1802): Tago, Inc., Burlingame, CA.

n. ABTS (2.2'-azinobis(3-ethylbenzthiazolinesulfonic acid)) solution:

Citric acid	100 mM
Na_2HPO_4	250 mM (pH 4.0/1 N HCl)
ABTS	0.5 mg/ml

ABTS solution should be kept in dark and 4°C. The solution should be discarded when it turns green.

o. Hydrogen Peroxide: 30% solution is kept in the dark and at 4°C.

Procedure. All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. Pat plate dry with paper towels.

A. Cell Seeding:

1. The cells, grown in tissue culture dish (Corning 25020-100) to 80-90% confluence, are harvested with Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO).

2. Resuspend the cells in fresh DMEM + 10% FBS + 2mM L-Glutamine, and transfer to 96 - well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 μl/well). Incubate for 1 day then replace medium to serum-free medium (90/μl) and incubate in 5% CO_2 and 37°C overnight.

B. ELISA Plate Coating and Blocking:

1. Coat the ELISA plate (Corning 25805-96) with Anti-IGF-1R Antibody at 0.5 μg/well in 100 μl PBS at least 2 hours.

2. Remove the coating solution, and replace with 100 μl Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

C. Assay Procedures:

1. The drugs are tested in serum-free condition.

2. Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well poly-propylene plate, and transfer 10 μl/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO_2 at 37°C for 2 hours.

3. Prepare fresh cell lysis buffer (HNTG*)

HNTG	2 ml
EDTA	0.1 ml
Na_3VO_4	0.1 ml
$Na_4(P_2O_7)$	0.1 ml
H_2O	7.3 ml

4. After drug incubation for two hours, transfer 10 μl/well of 200nM IGF-1 Ligand in PBS to the cells (Final Conc. = 20 nM), and incubate at 5% CO_2 at 37°C for 10 minutes.

5. Remove media and add 100μl/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

6. Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispense. Transfer all the lysate to the antibody coated ELISA plate, and shake for 1 hour.

7. Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

8. Remove anti-pTyr, wash the plate, transfer Tago (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

9. Remove detection antibody, wash the plate, and transfer fresh ABTS/H2O2 (1.2 μl H_2O_2 to 10 ml ABTS) 100 μl/well to the plate to start color development.

10. Measure OD in Dynatec MR5000, which is connected to Ingres.

(v) EGF Receptor ELISA

EGF Receptor kinase activity (EGFR-NIH3T3 assay) in whole cells was measured as described below:

Materials and Reagents. The following materials and reagents were used

a. EGF Ligand: stock concentration = 16.5 μM; EGF 201, TOYOBO, Co., Ltd. Japan.

b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

c. Anti-phosphotyosine antibody (anti-Ptyr) (polyclonal).

d. Detection antibody: Goat anti-rabbit lgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.

e. TBST buffer:

Tris-HCl, pH 7	50 mM
NaCl	150 mM
Triton X-100	0.1

f. HNTG 5X stock:

HEPES	0.1 M
NaCl	0.75 M
Glycerol	50
Triton X-100	1.0%

g. ABTS stock:

Citric Acid	100 mM
Na_2HPO_4	250 mM
HCl, conc.	4.0 pH
ABTS*	0.5 mg/ml

Keep solution in dark at 4°C until used.

h. Stock reagents of:

EDTA 100 mM pH 7.0
Na_3VO_4 0.5 M
$Na_4(P_2O_7)$ 0.2 M

Procedure. The following protocol was used:

A. <u>Pre-coat ELISA Plate</u>

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody at 0.5 μg per well in PBS, 150 μl final volume/well, and store overnight at

4°C. Coated plates are good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and replace with blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to use, remove blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. NIH 3T3/C7 cell line (Honegger, et al., Cell 51:199-209, 1987) can be use for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% CS DMEM medium. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1000 rpm, and once at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37°C for about 40 hours.

C. Assay Procedures.

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 μl to a test well for a final drug dilution of 1:200 and a

final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO_2 at 37°C for one hour.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μl dilute EGF (1:12 dilution), 25 nM final concentration is attained.

3. Prepare fresh 10 ml HNTG* sufficient for 100 μl per well wherein HNTG* comprises: HNTG stock (2.0 ml), milli-Q H_2O (7.3 ml), EDTA, 100 mM, pH 7.0 (0.5 ml), Na_3VO_4 0.5 M (0.1 ml) and Na_4 (P_2O_7), 0.2 M (0.1 ml).

4. Place on ice.

5. After two hours incubation with drug, add prepared EGF ligand to cells, 10 μl per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

6. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μl per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

7. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

8. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μl per well. Incubate shaking at room

temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

9. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO 30 anti-rabbit IgG antibody to the ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

10. Remove detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 μl per well. Incubate at room temperature for 20 minutes. ABTS/H_2O_2 solution: 1.2 μl 30% H_2O_2 in 10 ml ABTS stock.

11. Stop reaction by adding 50 μl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

12. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

(vi) Cellular Insulin Receptor ELISA

The following protocol was used to determine whether the compounds of the present invention possessed insulin receptor tyrosine kinase activity.

Materials And Reagents. The following materials and reagents were used to measure phophotyrosine levels

on the insulin receptor (indicating insulin receptor tyrosine kinase activity):

1. The preferred cell line was an NIH3T3 cell line (ATCC No. 1658) which overexpresses Insulin Receptor (H25 cells);

2. H25 cells are grown in an incubator with 5% CO_2 at 37°C. The growth media is DMEM + 10% FBS (heat inactivated) + 2mm L-Glutamine;

3. For ELISA plate coating, the monoclonal anti-IR antibody named BBE is purified and used;

4. D-PBS, comprising:

KH_2PO_4	0.20 g/l (GIBCO, 310-4190AJ)
K_2HPO_4	2.16 g/l
KCl	0.20 g/l
NaCl	8.00 g/l (pH 7.2);

5. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk);

6. TBST buffer, comprising:

Tris-HCl	50mM
NaCl	150mM pH 7.2 (HCI, 1 N)
Triton X-100	0.1%

Note: Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution;

7. HNTG buffer, comprising:

HEPES	20mM
NaCl	150mM pH 7.2 (HCI, 1 N)
Glycerol	10%
Triton X-100	0.2%

Note: Stock solution (5X) is prepared and kept at 4°C;

8. EDTA.HCI: 0.5 M pH 7.0 (NaOH) as 100X stock;

9. Na_3V0_4: 0.5 M as 100X stock and aliquots are kept in -80°C;

10. $Na_4P_20_7$: 0.2 M as 100X stock;

11. Insulin from GIBCO BRL (Cat# 18125039);

12. Polyclonal antiserum Anti-phosphotyrosine: rabbit sera or UB40 monoclonal antibody specific for phosphotyrosiene.

13. Detection antibody, preferably goat anti-rabbit IgG, POD conjugate, Tago (Cat. No. 4520: Lot No. 1802): Tago, Inc., Burlingame, CA;

14. ABTS solution, comprising:

Citric acid	100 mM
Na_2HP0_4	250 mM pH 4.0 (1 N HCI)
ABTS	0.5 mg/ml

wherein ABTS is 2,2'-azinobis (3-ethylbenathiazoline sulfonic acid) and stored in the dark at 4°C and discarded when it turns green;

15. Hydrogen Peroxide: 30% solution is kept in the dark and at 40°C.

Protocol. All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. All plates were tapped dry with paper towels prior to use.

A. Cell Seeding:

1. The cells were grown in tissue culture dish (10 cm, Corning 25020-100) to 80-90% confluence and

harvested with Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO);

2. Resuspend the cells in fresh DMEM + 10% FBS + 2mM L-Glutamine, and transfer to 96 - well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 μl/well). The cells are then incubated for 1 day. Following such incubation, 0.01% serum medium (90/μl) replaces the old media and the cells incubate in 5% CO_2 and 37°C overnight.

B. ELISA Plate Coating and Blocking:

1. Coat the ELISA plate (Corning 25805-96) with Anti-IR Antibody at 0.5 μg/well in 100 μl PBS at least 2 hours.

2. Remove the coating solution, and replace with 100 u; blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

C. Assay Procedures

1. The drugs are tested in serum-free condition.

2. Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well poly-propylene plate, and transfer 10 μl/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO_2 at 37°C for 2 hours.

3. Prepare fresh cells lysis buffer (HNTG*)

HNTG (5x)	2 ml
EDTA	0.1 ml

Na_3V0_4	0.1 ml
$Na_4P_20_7$	0.1 ml
H_20	7.3 ml
HNTG*	10 ml

4. After drug incubation for two hours, transfer 10 μl/well of 1μM insulin in PBS to the cells (Final concentration = 100 nM), and incubate at 5% $C0_2$ at 37°C for 10 minutes.

5. Remove media and add 100 μl/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

6. Using a 12-channel pipette, scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispense. Transfer all the lysate to the antibody coated ELISA plate, and shake for 1 hour.

7. Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

8. Remove anti-pTyr, wash the plate, transfer Tago (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

9. Remove detection antibody, wash the plate, and transfer fresh ABTS/H_20_2 (1.2 μl H_20_2 to 10 ml ABTS) 100 μl/well to the plate to start color development.

10. Measure OD in Dynatec MR5000, which is connected to Ingres. All following steps should follow Ingres instruction.

(vii) Purpose

To provide a consistent method for measuring the invitro kinase activity of the EGFR in an Enzyme-linked immunosorbent assay (Elisa).

Scope. The following protocol describes the procedures used to analyze protein tyrosine kinase activity on the EGFR in an Elisa. The procedure also describes the protocol for the initial screening of drugs for inhibition or activation of protein tyrosine kinase activity.

Reagents and Supplies.

1. Corning 96-well Elisa plates
   Corning Catalog #25805-96
2. 05-101 monoclonal anti-EGFR antibody (commercially available from UB1)
   -80 C, 1 ml aliquots
3. PBS (Dulbecco's Phosphate-Buffered Saline)
   Gibco Catalog # 450-1300EB
   Formulation: 2.7 mM KCL
   1.1 mM KH2PO4
   0.5 mM MgCl2 (anhydrous)
   138 mM NaCl
   8.1 mM Na2HPO4
4. TBST Buffer
   Formulation: 50 mM Tris pH 7.2
   150 mM NaCl
   0.1% Triton X-100

5. Blocking Buffer

Formulation: 5% Carnation Instant Milk in PBS

6. A431 cell lysate

A431 cells are available from a variety of commercial sources and may be used lysed using conventional methods known to those skilled in the art or as described for lysis of the 3T3 cells in the EGF cellular assay described herein. -80 C, 1 ml aliquots

7. TBS Buffer

Formulation: 50 mM Tris pH 7.2
150 mM NaCl

8. TBS + 10% DMSO

Formulation: 10% DMSO in TBS Buffer
(DMSO from Sigma, Catalog # D-2650)

9. ATP/MnCl2 phosphorylation mix

Formulation: 0.03 mM ATP
(Adenosine-5'-triphosphate,Sigma Catalog #A-5394)
50 mM MnCl2

Make fresh in autoclaved Milli-Q H2O immediately before use

Keep on ice until use

10. NUNC 96-well V bottom polypropylene plates

Applied Scientific Catalog # AS-72092

11. EDTA

Formulation: 200 mM EDTA pH 8.0

12. Rabbit polyclonal anti-phosphotyrosine serum or UB40 monoclonal antibody specific for phosphotyrosine

- 80 C, 1 ml aliquots

Thaw 1 ml vial and aliquot in smaller volumes to store at - 80 C

Antiserum is stable for weeks when thawed and stored at 4 C

13. Goat anti-rabbit IgG peroxidase conjugate

Biosource Catalog # ALI0404

14. ABTS Solution

Formulation: 100 mM Citric Acid (anhydrous)
250 mM Na2HPO4 pH 4.0
0.5 mg/ml ABTS

(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

(Sigma Catalog # A-1888)

Keep solution in dark at 4 C until ready to use

15. Hydrogen peroxide 30% solution

Fisher Catalog # H325

Store in the dark at 4 C until ready to use

16. ABTS/H2O2

Formulation: 15 mls ABTS solution
2 ul H2O2

Prepare 5 minutes before use and room temperature

17. 0.2 M HCL stock in H2O

Procedure.

1. Coat Corning 96-well elisa plates with 0.5 ug per well 05-101 antibody.

Bring final volume to 100 ul per well with PBS.

Coat plates overnight at 4 C.

2. Remove unbound 05-101 from wells by inverting plate to remove liquid.

Wash 1x with distilled H2O by filling wells

Pat the plate on a paper towel to remove excess liquid.

3. Block plates with 5% milk in PBS.

150 ul per well.

Incubate plate 30 minutes while shaking on a microtiter plate shaker.

4. Wash plate 3x with dionized water, then once with TBST

5. Add 7 ug A431 cell lysate per well (EGFR source).

Add PBS to final volume of 100 ul per well

Incubate 30 minutes while shaking.

6. Wash as described in step 4.

7. At this point, drugs or extracts are added to the wells.

Dilute drugs/extracts 1:100 (unless specified otherwise) in TBS + 10% DMSO in 96-well polypropylene plates.

Add 120 ul TBS to ELISA plate containing captured EGFR.

Add 13.5 ul diluted drugs/extracts to ELISA plate.

To control wells (wells which do not receive any drug) add 135 ul TBS

+ 1% DMSO.

Incubate plate 30 minutes while shaking.

8. Add 15 ul of 0.03 mM ATP + 50 mM MnCl2 phosphorylation mix directly to all wells except negative control well which does not receive ATP/MnCl2 (see diagram).

(150 ul final volume in well with 3 uM ATP/5 mM MnCl2 final concentration in well.)

Incubate 5 minutes while shaking vigorously.

*NOTE: It is critical that ATP/MnCl2 phosphorylates the receptor for 5 minutes only. It is best to add the ATP/MnCl2 with an 12 channel pipettor 1 row at a time leaving 20 seconds between each row so that the reaction may be stopped with EDTA exactly 5 minutes later (this depends on the number of plates being phosphorylated in one batch). Shake between each addition.

9. After 5 minutes, to stop reaction, add 16.5 ul of 200 mM EDTA pH 8.0 for 20 mM final in well, shaking continuously between each addition. This is done using the same timing method as above. After last row has received EDTA, shake plate an additional minute.

10. Wash 4x with deionized water, twice with TBST.

11. Add rabbit polyclonal anti-phosphotyrosine serum.

Dilute 1:3000 in TBST.

Add 100 ul per well.

Incubate 30-45 minutes while shaking.

12. Wash as described above in step 4.

13. Add BioSource anti-rabbit peroxidase conjugate antibody.

Dilute 1:2000 in TBST.

Add 100 ul per well.

Incubate 30 minutes while shaking.

14. Wash as described in step 4.

15. Add 100 ul of ABTS/H2O2 solution to well.

Incubate 5 to10 minutes while shaking.

Remove bubbles

16. If necessay stop reaction with the addition of 100ul of 0.2M HCl per well

17. Read assay on Dynatech MR7000 elisa reader.

Test Filter: 410 nM

Reference Filter: 630 nM

(b) Cell Growth Assays

The following assays may be conducted to measure the effect of the claimed compounds upon cell growth as a result of the compound's interaction with one or more RTKs. Unless otherwise specified, the following assays may be generally applied to measure the activity of a compound against any particular RTK. To the extent that an assay, set forth below, refers to a specific RTK, one skilled in the art would be able to adapt the disclosed protocol for use to measure the activity of a second RTK.

(i) Soft Agar Assay

The soft agar assay may be used to measure the effects of substances on cell growth. Unless otherwise stated the soft agar assays were carried out as follows:

Material And Reagents. The following materials and reagents were used:

a. A water bath set at 39°C and another water bath at 37°C.

b. 2X assay medium is comprised of 2X Dulbecco's 5Modified Eagle's Medium (DMEM) (Gibco Cat. # CA400-4ANO3) supplemented by the following:

- 20% Fetal Bovine Serum (FBS) 2 mM sodium pyruvate 4 mM glutamine amine; and
- 20 mM HEPES Non-essential Amino Acids (1:50 from lOOx stock).

c. 1X assay medium made of 1X DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES, non-essential amino acid (1:100 from lOOx stock).

d. 1.6% SeaPlaque Agarose in autoclave bottle.

e. Sterile 35 mm Corning plates (FMC Bioproducts Cat. #50102).

f. Sterile 5 ml glass pipets (individually wrapped).

g. Sterile 15 ml and 50 ml conical centrifuge tubes.

h. Pipets and sterile tips.

i. Sterile microcentrifuge tubes.

j. Cells in T75 flasks: SKOV-3 (ATCC HTB77).

k. 0.25% Trypsin solution (Gibco #25200-015).

Procedure. The following procedure was used to conduct the soft agar assay:

A. Procedure for making the base layer

1. Have all the media warmed up in the 37°C water bath.

2. To make 1X of assay medium + 0.8% agar: make a 1:2 (vol:vol) dilution of melted agar (cooled to 39°C), with 2X assay medium.

3. Keep all media with agar warm in the 39°C water bath when not in use.

4. Dispense 1 ml of 1X assay medium + 0.8% agar into dishes and gently swirl plate to form a uniform base layer. Bubbles should be avoided.

5. Refrigerate base layers to solidify (about 20 minutes). Base layers can be stored overnight in the refrigerator.

B. Procedure for collecting cells

1. Take out one flask per cell line from the incubator; aspirate off medium; wash once with PBS and aspirate off; add 3 ml of trypsin solution.

2. After all cells dissociate from the flask, add 3 ml of 1X assay media to inhibit trypsin activity. Pipet the cells up and down, then transfer the suspension into a 15ml tube.

3. Determine the concentration of cells using a Coulter counter, and the viability by trypan blue exclusion.

4. Take out the appropriate volume needed to seed 3300 viable cells per plate and dilute it to 1.5 ml with 1X assay medium.

C. Procedure for making the upper 0.4% agarose layer:

1. Add TBST compounds at twice the desired final assay concentration; + 1.5 ml of cell suspension in 1X assay medium 10% FBS; + 1.5 ml of 1X assay medium + 0.8% agarose*: Total = 3.0 ml 1X media 10% FBS + 0.4% agarose with 3300 viable cells/ml, with and without TBST compounds.

*(Made by 1:2 dilution of 2X media with 1.6% agar 30 for the base layer procedure above.)

2. Plate 1 ml of the Assay Mix onto the 1 ml base layer. The duplicates are plated from the 3 ml volume.

3. Incubate the dishes for 2-3 weeks in a 100% humidified, 10% CO_2 incubator.

4. Colonies that are 60 microns and larger are scored positive.

(ii) Sulforhodamine B (SRB) Growth Assays

The SRB assays may be used to measure the effects of substances or cell growth. The assays are carried out as follows:

Assay 1: 3T3/E/H+TGF-a(T) Cell Growth SRB Assay

Materials:

96-well flat bottom sterile plates

96-well round bottom sterile plates

sterile 25 ml or 100 ml reservoir
pipets, multi-channel pipetman
sterile pipet tips
sterile 15 ml and 50 ml tubes

Reagents:
0.4% SRB in 1% acetic acid
10 mM Tris base
10% TCA
1% acetic acid
sterile DMSO (Sigma)
compound in DMSO (100 mM or less stock solution)
25% Trypsin-EDTA in Cell Dissociation Solution (Sigma)

Cell line and growth medium:
3T3/E/H+TGF-a(T) (NIH 3T3 clone 7 cells expressing EGF-R/HER2 chimera and TGF-a, tumor-derived autocrine loop cells)
2% calf serum/DMEM + 2 mM glutamine

Protocol:
Day 0: Cell Plating:
This part of assay is carried out in a laminar flow hood.

1. Trypsinize cells as usual. Transfer 100 μl of cell suspension to 10 ml of isotone. Count cells with the Coulter Counter.

2. Dilute cells in growth medium to 60,000 cells/ml. Transfer 100 μl of cells to each well in a 96-well flat bottom plate to give 6000 cells/well.

3. Use half of plate (4 rows) for each compound and quadruplicate wells for each compound concentration, a set of 4 wells for medium control and 4 wells for DMSO control.

4. Gently shake plates to allow for uniform attachment of the cells.

5. Incubate the plates at 37°C in a 10% CO2 incubator.

Day 1: Addition of Compound:
This part of assay is carried out in a laminar flow hood.

1. In 96 well-round bottom plate, add 125 μl of growth medium to columns 3 to 11. This plate is used to titrate out the compound, 4 rows per compound.

2. In a sterile 15 ml tube, make a 2X solution of the highest concentration of compound by adding 8 μl of the compound to a total of 2 ml growth medium for a dilution of 1:250. At this dilution, the concentration of DMSO is 0.4% for a 2X solution or 0.2% for 1X solution on the cells. The starting concentration of the compound is usually 100 uM but this concentration may vary depending upon the solubility of the compound.

3. Transfer the 2X starting compound solution to quadruplicate wells in column 12 of the 96-well round bottom plate. Do 1:2 serial dilutions across the plate from right to left by transferring 125 μl from column 12 to column 11, column 11 to 10 and so on. Transfer 100 μl of compound dilutions onto 100 μl medium on cells in corresponding wells of 96-well flat bottom plate. Total volume per well should be 200 μl.

4. For vehicle control, prepare a 2X solution of DMSO at 0.4% DMSO in growth medium. Transfer 100 μl of the DMSO solution to the appropriate wells of cells. The final concentration of DMSO is 0.2%.

5. For the medium control wells, add 100 μl/well of growth medium to the appropriate wells of cells.

6. Return the plate to the incubator and incubate for 4 days.

Day 5: Development of Assay

This part of assay is carried out on the bench.

1. Aspirate or pour off medium. Add 200 μl cold 10% TCA to each well to fix cells. Incubate plate for at least 60 min. at 4°C.

2. Discard TCA and rinse wells 5 times with water. Dry plates upside down on paper towels.

3. Stain cells with 100 μl/well 0.4% SRB for 10 min.

4. Pour off SRB and rinse wells 5 times with 1% acetic acid. Dry plates completely upside down on paper towels.

5. Solubilize dye with 100 μl/well 10 mM Tris base for 5-10 min. on shaker.

6. Read plates on Dynatech ELISA Plate Reader at 570 nm with reference at 630 nm.

Assay 2: 3T3/EGF-R+TGF-a(T) Cell Growth SRB Assay

Materials and Reagents same as for Assay 1.
Cell line and growth medium:
3T3/EGF-R+TGF-a(T) (NIH 3T3 clone 7 cells expressing EGF-R and TGF-a, tumor-derived autocrine loop cells)
2% calf serum/DMEM + 2 mM glutamine

Protocol:
Day 0: Cell Plating:
This part of assay is carried out in a laminar flow hood.

1. Trypsinize cells as usual. Transfer 100 μl of cell suspension to 10 ml of isotone. Count cells with the Coulter Counter.

2. Dilute cells in growth medium to 60,000 cells/ml. Transfer 100 μl of cells to each well in a 96-well flat bottom plate to give 6000 cells/well.

3. Use half of plate (4 rows) for each compound and quadruplicate wells for each compound concentration,

a set of 4 wells for medium control and 4 wells for DMSO control.

4. Gently shake plates to allow for uniform attachment of the cells.

5. Incubate the plates at 37°C in a 10% CO2 incubator.

Day 1: Addition of Compound: same as for Assay 1.

Day 5: Development of Assay: same as for Assay 1.

Assay 3: 3T3/PDGF-βR/PDGF-BB(T) Cell Growth SRB Assay

Cell line and growth medium:

3T3/PDGF-βR/PDGF-BB(T) (NIH 3T3 clone 7 cells expressing PDGF β-receptor and PDGF-BB, from tumors resected from athymic mice)

2% calf serum/DMEM + 2 mM glutamine

Protocol:

Day 0: Cell Plating:

This part of assay is carried out in a laminar flow hood.

1. Trypsinize cells as usual. Transfer 200 μl of cell suspension to 10 ml of isotone. Count cells on the Coulter Counter.

2. Dilute cells in growth medium to 60,000 cells/ml. Transfer 100 μl of cells to each well in a 96-well flat bottom plate to give 6000 cells/well.

3. Allow half of plate (4 rows) for each compound and quadruplicate wells for each compound concentration, a set of 4 wells for medium control and 4 wells for DMSO control.

4. Gently shake plates to allow for uniform attachment of the cells to the plate.

5. Incubate the plates at 37°C in a 10% CO_2 incubator.

Day 1: Addition of Compound: same as for Assay 1.

Day 5: Development of Assay: same as for Assay 1.

Assay 4: Human Smooth Muscle Cells (SMC) Growth SRB Assay

Materials and Reagents same as for Assay 1:

Cell line and growth medium:

Human Aortic Smooth Muscle cells (Clonetics)

Clonetics's Bullet Kit: Smooth Muscle Basal Medium (SmBM) which is modified MCDB 131 containing fetal bovine serum (5%), hFGF (2ng/ml), hEGF (0.1 ng/ml), insulin (5.0 ug/ml), gentamicin (50ug/ml) and amphotericin B (50 ng/ml)

Protocol:

Day 0: Cell plating:

This part of assay is carried out in a laminar flow hood.

1. Trypsinize cells as usual. Transfer 200 μl of cell suspension to 10 ml of isotone. Count cells on the Coulter Counter.

2. Dilute cells in growth medium to 20,000 cells/ml. Transfer 100 μl of cells to each well in a 96-well flat bottom plate to give 2000 cells/well.

3. Allow half of plate (4 rows) for each compound and quadruplicate wells for each compound concentration, a set of 4 wells for medium control and 4 wells for DMSO control.

4. Gently shake plates to allow for uniform attachment of the cells to the plate.

5. Incubate the plates at 37°C in a 10% CO2 incubator.

Day 1: Addition of Compound: same as for Assay 1.

Day 5: Development of Assay: same as for Assay 1.

(iii) 3T3 Cell Growth Assay

Assay 1: PDGF-Induced BrdU Incorporation Assay

Materials and Reagents:

(1) PDGF: human PDGF B/B; 1276-956, Boehringer Mannheim, Germany

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

Protocol

(1) 3T3 engineered cell line: 3T3/EGFRc7.

(2) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(3) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(4) On day 3, ligand (PDGF=3.8 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (PDGF) but no test

compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(5) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.

(6) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(7) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(8) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 μl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(9) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times

with PBS, and the plate is dried by inverting and tapping on a paper towel.

(10) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(11) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 2: EGF-Induced BrdU Incorporation Assay

Materials and Reagents

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

Protocol

(1) 3T3 engineered cell line: 3T3/EGFRc7

(2) Cells are seeded at 8000 cells/well in 10% CS, 2mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(3) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(4) On day 3, ligand (EGF=2 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

5) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.

6) After incubation with labeling reagent, the medium is removed by decanting and tapping the

inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(7) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(8) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(9) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(10) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(11) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 3: EGF-Induced Her2 -Driven BrdU Incorporation

Materials and Reagents:

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

Protocol:

(1) 3T3 engineered cell line: 3T3/EGFr/Her2/EGFr (EGFr with a Her2 kinase domain)

(2) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96- well plate. Cells are incubated overnight at 37° in 5% CO_2.

(3) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(4) On day 3, ligand (EGF=2 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

5) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.

(6) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(7) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(8) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1%

BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(9) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(10) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(11) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 4: IGF1-Induced BrdU Incorporation Assay

Materials and Reagents:

(1) IGF1 Ligand: human, recombinant; G511, Promega Corp, USA.

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

Protocol:

(1) 3T3 engineered cell line: 3T3/IGF1r.

(2) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96- well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(3) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(4) On day 3, ligand (IGF1=3.3 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (IGF1) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

5) After 16 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.

(6) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(7) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(8) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 μl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(9) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(10) TMB substrate solution is added (100 μl/well) and incubated for 20 minutes at room

temperature on a plate shaker until color development is sufficient for photometric detection.

(11) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 5: Insulin-Induced BrdU Incorporation Assay

Materials and Reagents:

(1) Insulin: crystalline, bovine, Zinc; 13007, Gibco BRL, USA.

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

Protocol:

(1) 3T3 engineered cell line: H25

(2) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(3) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(4) On day 3, ligand (Insulin=10 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (Insulin) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(5) After 16 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.

(6) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(7) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(8) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(9) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(10) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(11) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

(iv) HUV-EC-C Assay

The following protocol may also be used to measure a compound's activity:

DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, (American Type Culture Collection; catalogue no. 1730 CRL). Wash with Dulbecco's phosphate-buffered saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029) 2 times at about 1 ml/10 cm^2 of tissue culture flask. Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company; catalogue no. C-1544). The 0.05% trypsin was made by diluting 0.25% trypsin/1 mM EDTA (Gibco; catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 ml/25-30 cm^2 of tissue culture flask for about 5 minutes at 37°C. After cells have detached from the flask, add an equal volume of assay medium and transfer to a 50 ml sterile centrifuge tube (Fisher Scientific; catalogue no. 05-539-6).

2. Wash the cells with about 35 ml assay medium in the 50 ml sterile centrifuge tube by adding the assay medium, centrifuge for 10 minutes at approximately 200xg, aspirate the supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two more times with D-PBS, resuspend the cells in about 1 ml assay medium/15 cm^2 of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL; catalogue no. 21127-014) + 0.5% heat-inactivated fetal bovine serum. Count the cells with a

Coulter Counter®v Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of $0.8\text{-}1.0\text{x}10^5$ cells/ml.

3. Add cells to 96-well flat-bottom plates at 100 µl/well or $0.8\text{-}1.0\text{x}10^4$ cells/well; incubate ~24h at 37°C, 5% CO_2.

DAY 1

1. Make up two-fold drug titrations in separate 96-well plates, generally 50 µM on down to 0 µM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 µl/well of drug at 200 µM (4X the final well concentration) to the top well of a particular plate column. Since the stock drug concentration is usually 20 mM in DMSO, the 200 µM drug concentration contains 2% DMSO.

Therefore, diluent made up to 2% DMSO in assay medium (F12K + 0.5% fetal bovine serum) is used as diluent for the drug titrations in order to dilute the drug but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 µl/well. Take 60 µl from the 120 µl of 200 µM drug dilution in the top well of the column and mix with the 60 µl in the second well of the column. Take 60 µl from this well and mix with the 60 µl in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 µl of the 120 µl in this well and discard it. Leave the last well with 60 µl of DMSO/media diluent as a non-drug-containing control. Make 9 columns of

titrated drug, enough for triplicate wells each for 1) VEGF (obtained from Pepro Tech Inc., catalogue no. 100-200, 2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained from Boehringer Mannheim Biochemica, catalogue no. 1439 600), and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 μl/well of the drug dilutions to the 96-well assay plates containing the 0.8-1.0x10^4 cells/100 μl/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37°C, 5% CO_2.

3. In triplicate, add 50 μl/well of 80 μg/ml VEGF, 20 ng/ml ECGF, or media control to each drug condition. As with the drugs, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24 hours at 37°C, 5% CO_2. Each well will have 50 μl drug dilution, 50 μl growth factor or media, and 100 ul cells, = 200 ul/well total. Thus the 4X concentrations of drugs and growth factors become 1X once everything has been added to the wells.

DAY 2

1. Add ^{3}H-thymidine (Amersham; catalogue no. TRK-686) at 1 μCi/well (10 μl/well of 100 μCi/ml solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37°C, 5% CO_2. Note: ^{3}H-thymidine is made up in RPMI media because all of the other applications for which we use the ^{3}H-thymidine

involve experiments done in RPMI. The media difference at this step is probably not significant. RPMI was obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at -20°C.

DAY 4

1. Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96(R)) onto filter mats (Wallac; catalogue no. 1205-401); read counts on a Wallac Betaplate(TM) liquid scintillation counter.

(v) PDGF-R Cellular Assay

The PDGF cellular kinase assay was carried out as follows: cells are lysed in 0.2 M Hepes, 0.15 M NaCl, 10% V/V glycerol, 0.04% Triton X-100, 5 mM EDTA, 5 mM sodium vanadate and 2 mM Na+ pyrophosphate; cell lysates are then added to an ELISA plate coated with an anti-PDGF receptor antibody (Genzyme); ELISA plates are coated at 0.5 μg of antibody/well in 150 μl of PBS for 18 hours at 4°C prior to the addition of the lysate; the lysate is incubated in the coated plates for 1 hour and then washed four times in TBST (35 mM Tris-HCl pH 7.0, 0.15 M NaCl, 0.1% Triton X100); anti-phosphotyrosine antibody (100 μl in PBS) is added and the mixture is incubated for 30 minutes at room temperature; the wells were then washed four times in TBST, a secondary antibody conjugated to POD (TAGO) is added to each well, and the treated well are incubated for 30 minutes at room temperature; the wells are then washed four times

in TBST, ABTS/H_2O_2 solution is added to each well and the wells are incubated for two minutes; absorbance is then measured at 410 nm.

(vi) Assay Measuring Phosphorylating Function of Raf

The following assay reports the amount of RAF-catalyzed phosphorylation of its target protein MEK as well as MEK's target MAPK. The RAF gene sequence is described in Bonner et al., 1985, *Molec. Cell. Biol. 5*: 1400-1407, and is readily accessible in multiple gene sequence data banks. Construction of the nucleic acid vector and cell lines utilized for this portion of the invention are fully described in Morrison et al., 1988, *Proc. Natl. Acad. Sci. USA 85*: 8855-8859.

Materials and Reagents

1. Sf9 (*Spodoptera frugiperda*) cells; GIBCO-BRL, Gaithersburg, MD.

2. RIPA buffer: 20 mM Tris/HCl pH 7.4, 137 mM NaCl, 10 % glycerol, 1 mM PMSF, 5 mg/L Aprotenin, 0.5 % Triton X-100;

3. Thioredoxin-MEK fusion protein (T-MEK): T-MEK expression and purification by affinity chromatography were performed according to the manufacturer's procedures. Catalog# K 350-01 and R 350-40, Invitrogen Corp., San Diego, CA

4. His-MAPK (ERK 2); His-tagged MAPK was expressed in XL1 Blue cells transformed with pUC18

vector encoding His-MAPK. His-MAPK was purified by Ni-affinity chromatography. Cat# 27-4949-01, Pharmacia, Alameda, CA

5. Sheep anti mouse IgG: Jackson laboratories, West Grove, PA Catalog, # 515-006-008, Lot# 28563

6. Sumo 22: Monoclonal antibody Sumo 22 (URP 30 S 3) specific for the RAF-1 protein kinase

7. Coating buffer: PBS; phosphate buffered saline, GIBCO-BRL, Gaithersburg, MD

8. Wash buffer: TBST - 50 mM Tris/HCL pH 7.2, 150 mM NaCl, 0.1 % Triton X-100

9. Block buffer: TBST, 0.1 % ethanolamine pH 7.4

10. DMSO, Sigma, St. Louis, MO

11. Kinase buffer (KB): 20 mM Hepes/HCl pH 7.2, 150 mM NaCl, 0.1 % Triton X-100, 1 mM PMSF, 5 mg/L Aprotenin, 75 μM sodium ortho vanadate, 0.5 mM DTT and 10 mM $MgCl_2$.

12. ATP mix: 100 mM $MgCl_2$, 300 μM ATP, 10 μCi γ-^{33}P ATP (Dupont-NEN)/mL.

13. Stop solution: 1 % phosphoric acid; Fisher, Pittsburgh, PA.

14. Wallac Cellulose Phosphate Filter mats; Wallac, Turku, Finland.

15. Filter wash solution: 1 % phosphoric acid, Fisher, Pittsburgh, PA.

16. Tomtec plate harvester, Wallac, Turku, Finland.

17. Wallac beta plate reader # 1205, Wallac, Turku, Finland.

18. NUNC 96-well V bottom polypropylene plates for compounds Applied Scientific Catalog # AS-72092.

Procedure

All of the following steps are conducted at room temperature unless specifically indicated.

1. ELISA plate coating: ELISA wells are coated with 100 μL of Sheep anti mouse affinity purified antiserum (1μg/100μL coating buffer) over night at 4 °C. ELISA plates can be used for two weeks when stored at 4 °C.

2. Invert the plate and remove liquid. Add 100 μL of blocking solution and incubate for 30 min.

3. Remove blocking solution and wash four times with wash buffer. Pat the plate on a paper towel to remove excess liquid.

4. Add 1 μg of purified Sumo 22 to each well and incubate for 1 hour. Wash as described in step 3.

5. Thaw lysates from RAS/RAF infected Sf9 cells and dilute with TBST to 10 μg/100 μL. Add 10 μg of diluted lysate to the wells and incubate for 1 hour. Shake the plate during incubation. Negative controls receive no lysate. Lysates from RAS/RAF infected Sf9 insect cells are prepared after cells are infected with recombinant baculoviruses at a MOI of 5 for each virus, and harvested 48 hours later. The cells are washed once

with PBS and lysed in RIPA buffer. Insoluble material is removed by centrifugation (5 min at 10 000 x g). Aliquots of lysates are frozen in dry ice/ethanol and stored at - 80 °C until use.

6. Remove non-bound material and wash as outlined above (step 3).

7. Add 2 μg of T-MEK and 2 μg of His-MAPK per well and adjust the volume to 40 μL with kinase buffer.

8. Predilute compounds (stock solution 10 mg/mL DMSO) or extracts 20 fold in TBST plus 1% DMSO. Add 5 μL of the prediluted compounds/extracts to the wells described in step 6. Incubate for 20 min. Controls receive no drug.

9. Start the kinase reaction by addition of 5 μL ATP mix; Shake the plates on an ELISA plate shaker during incubation.

10. Stop the kinase reaction after 60 min by addition of 30 μL stop solution to each well.

11. Place the phosphocellulose mat and the ELISA plate in the Tomtec plate harvestor. Harvest and wash the filter with the filter wash solution according to the manufacturers recommendation. Dry the filter mats. Seal the filter mats and place them in the holder. Insert the holder into radioactive detection apparatus and quantitate the radioactive phosphorous on the filter mats.

Alternatively, 40 μL aliquots from individual wells of the assay plate can be transferred to the

corresponding positions on the phosphocellulose filter mat. After air-drying the filters, put the filters in a tray. Gently rock the tray, changing the wash solution at 15 min intervals for 1 hour. Air-dry the filter mats. Seal the filter mats and place them in a holder suitable for measuring the radioactive phosphorous in the samples. Insert the holder into a detection device and quantitate the radioactive phosphorous on the filter mats.

(c) Measurement Of Cell Toxicity

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: IC_{50}/LD_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques (Mossman, 1983, *J. Immunol. Methods*, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, *J. Immunol. Methods* 64:313; Decker and Lohmann-Matthes, 1988, *J. Immunol. Methods* 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

EXAMPLE 3: *IN VIVO* ANIMAL MODELS

(i) Xenograft Animal Models

The ability of human tumors to grow as xenografts in athymic mice (*e.g.*, Balb/c, nu/nu) provides a useful in vivo model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, *Acta Pathol. Microbial. Scand.* 77:758-760), many different human tumor cell lines (*e.g.*, mammary, lung, genitourinary, gastrointestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. Human mammary tumor cell lines, including MCF-7, ZR75-1, and MDA-MB-231, have been established as subcutaneous xenografts in nude mice (Warri *et al.*, 1991, *Int. J. Cancer* 49:616-623; Ozzello and Sordat, 1980, *Eur. J. Cancer* 16:553-559; Osborne *et al.*, 1985, *Cancer Res.* 45:584-590; Seibert *et al.*, 1983, *Cancer Res.* 43:2223-2239).

Assay 1: HER2/Xenograft Animal Model

To study the effect of anti-tumor drug candidates on HER2 expressing tumors, the tumor cells should be able to grow in the absence of supplemental estrogen. Many mammary cell lines are dependent on estrogen for in vivo growth in nude mice (Osborne *et al.*, *supra*), however, exogenous estrogen suppresses HER2 expression in nude mice (Warri *et al.*, *supra*, Dati *et al.*, 1990,

Oncogene 5:1001-1006). For example, in the presence of estrogen, MCF-7, ZR-75-1, and T47D cells grow well *in vivo*, but express very low levels of HER2 (Warri *et al.*, *supra*, Dati *et al.*, *supra*).

The following type of xenograft protocol can be used:

1) implant tumor cells (subcutaneously) into the hindflank of five- to six-week-old female Balb/c nu/nu athymic mice;
2) administer the anti-tumor compound;
3) measure tumor growth by measuring tumor volume.

The tumors can also be analyzed for the presence of a receptor, such as HER2, EGF or PDGF, by Western and immunohistochemical analyses. Using techniques known in the art, one skilled in the art can vary the above procedures, for example through the use of different treatment regimes.

Assay 2: FLK-1/Xenograft Model.

The ability of the compounds of the present invention to inhibit ovarian, melanoma, prostate, lung and mammary tumor cell lines established as SC xenografts was examined. These studies were conducted using doses ranging from 1 to 75 mg/kg/day.

Materials And Methods. The tumor cells were implanted subcutaneously into the indicated strains of mice. Treatment was initiated on day 1 post implantation unless otherwise indicated (*e.g.* treatment

of the SCID mouse related to the A375 melanoma cell line began on Day 9). Eight (8) to sixteen (16) mice comprised each test group.

Specifically:

Animals. Female athymic mice (BALB/c, nu/nu), BALB/c mice, Wistar rats and Fisher 344 rats were obtained from Simonsen Laboratories (Gilroy, CA). Female A/I mice were obtained from Jackson Laboratory (Bar Harbor, ME). DA rats were obtained from B&K Universal, Inc. (Fremont, CA). Athymic R/Nu rats, DBA/2N mice, and BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Female C57BL/6 mice were obtained from Taconic (Germantown, NY). All animals were maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They received sterile rodent chow and water *ad libitum*.

All procedures were conducted in accordance with the *NIH Guide for the Care and Use Of Laboratory Animals*.

Subcutaneous Xenograft Model. Cell lines were grown in appropriate medium as described (See Section 6). Cells were harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets were resuspended in sterile PBS or media (without FBS) to a suitable concentration indicated in the Figure legends and the cells were implanted into the hindflank of mice. Tumor growth was measured over 3 to 6 weeks using venier calipers tumor volumes were calculated as a product of length x width x height unless otherwise indicated. P values were calculated

using the Students' t-test. Compound in 50 - 100 μL excipient (dimethylsulfoxide, PBTE, PBTE6C:D5W, or PBTE:D5W) was delivered by IP injection at concentrations indicated in the Figure legends.

Intracerebral Xenograft Model. For the mouse IC model, rat C6 glioma cells were harvested and suspended in sterile PBS at a concentration of 2.5×10^7 cells/ml and placed on ice. Cells were implanted into BALB/c, nu/nu mice in the following manner: the frontoparietal scalps of mice were shaved with animal clippers if necessary before swabbing with 70% ethanol. Animals were anesthetized with isofluorane and the needle was inserted through the skull into the left hemisphere of the brain. Cells were dispensed from Hamilton Gas-tight Syringes using 30 ga ½ inch needles fitted with sleeves that allowed only a 3 mm penetration. A repeater dispenser was used for accurate delivery of 4 μL of cell suspension. Animals were monitored daily for well-being and were sacrificed when they had a weight loss of about 40% and/or showed neurological symptoms.

For the rat IC model, rats (Wistar, Sprague Dawley, Fisher 344, or athymic R/Nu; approximately 200-400 g (some 3-400g)) were anesthetized by an IP injection of 100 mg/kg Ketaset (ketamine hydrochloride; Aveco, Fort Dodge, Iowa) and 5 mg/kg Rompun (xylazine, 2% solution; Bayer, Germany). After onset of anesthesia, the scalp was shaved and the animal was oriented in a stereotaxic apparatus (Stoelting, Wood Dale, IL). The skin at the

incision site was cleaned 3 times with alternating swabs of 70% ethanol and 10% Povidone-Iodine. A median 1.0 - 1.5 cm incision was made in the scalp using a sterile surgical blade. The skin was detached slightly and pulled to the sides to expose the sutures on the skull surface. A dental drill (Stoelting, Wood Dale, IL) was used to make a small (1-2 mm diameter) burrhole in the skull approximately 1 mm anterior and 2 mm lateral to the bregma. The cell suspension was drawn into a 50 μL Hamilton syringe fitted with a 23 or 25g a standard bevel needle. The syringe was oriented in the burrhole at the level of the arachnoidea and lowered until the tip of the needle was 3 mm deep into the brain structure, where the cell suspension was slowly injected. After cells were injected, the needle was left in the burrhole for 1-2 minutes to allow for complete delivery of the cells. The skull was cleaned and the skin was closed with 2 to 3 sutures. Animals were observed for recovery from surgery and anesthesia. Throughout the experiment, animals were observed at least twice each day for development of symptoms associated with progression of intracerebral tumor. Animals displaying advanced symptoms (leaning, loss of balance, dehydration, loss of appetite, loss of coordination, cessation of grooming activities, and/or significant weight loss) were humanely sacrificed and the organs and tissues of interest were resected.

Intraperitoneal Model. Cell lines were grown in the appropriate media. Cells were harvested and

washed in sterile PBS or medium without FBS, resuspended to a suitable concentration, and injected into the IP cavity of mice of the appropriate strain. Mice were observed daily for the occurrence of ascites formation. Individual animals were sacrificed when they presented with a weight gain of 40%, or when the IP tumor burden began to cause undue stress and pain to the animal.

(ii) *In Vivo* VEGF Pellet Model

In the following example, the Pellet Model was used to test a compound's activity against the FLK-1 receptor and against disorders associated with the formation of blood vessels. In this model, VEGF is packaged into a time-release pellet and implanted subcutaneously on the abdomen of nude mice to induce a `reddening' response and subsequent swelling around the pellet. Potential FLK-1 inhibitors may then be implanted in methylcellulose near the VEGF pellet to determine whether such inhibitor may be used to inhibit the "reddening" response and subsequent swelling.

Materials And Methods. The following materials were used:

1) VEGF- human recombinant lyophilized product is commercially may be obtained from Peprotech, Inc., Princeton Business Park, G2; P.O. box 275, Rocky Hill, NJ 08553.

2) VEGF packaged into 21 day release pellets were obtained from Innovative Research of America (Innovative Research of America, 3361 Executive Parkway, P.O. Box

2746, Toledo, Ohio 43606), using patented matrix driven delivery system. Pellets were packaged at 0.20, 0.21, or 2.1 μg VEGF/pellet. These doses approximate 10 and 100 ng/day release of VEGF.

3) Methylcellulose

4) Water (sterile)

5) Methanol

6) Appropriate drugs/inhibitors

7) 10 cm culture plates

8) parafilm

The following protocol was then followed to conduct the VEGF pellet model:

1) VEGF, purchased from Peprotech, was sent to Innovative Research for Custom Pellet preparation;

2) Methylcellulose prepared at 1.5% (w/v) in sterile water;

3) Drugs solubilized in methanol (usual concentration range = 10 to 20 mg/ml);

4) Place sterile parafilm in sterile 10 cm plates;

5) 150 μl of drug in methanol added to 1.35 ml of 1.5% methylcellulose and mixed/vortexed thoroughly;

6) 25 μl aliquots of homogenate placed on parafilm and dried into discs;

7) Mice (6-10 wk. Balb/C athymic nu/nu, female) were anesthetized via isoflurane inhalation; 8) VEGF pellets and methylcellulose discs were implanted subcutaneously on the abdomen; and

9) Mice were scored at 24 hours and 48 hours for reddening and swelling response.

The specific experimental design used in this example was:

N = 4 animals/group

Controls: VEGF pellet + drug placebo

VEGF placebo + drug pellet

(iii) Mammary Fat Pad Model

Because of the established role played by many of the RTKs, *e.g.*, the HER2 receptor, in breast cancer, the mammary fat pad model is particularly useful for measuring the efficacy of compounds which inhibit such RTKs. By implanting tumor cells directly into the location of interest, *in situ* models more accurately reflect the biology of tumor development than do subcutaneous models. Human mammary cell lines, including MCF-7, have been grown in the mammary fat pad of athymic mice. Shafie and Grantham, 1981, *Natl. Cancer Instit.* 67:51-56; Gottardis *et al.*, 1988, *J. Steroid Biochem.* 30:311-314. More specifically, the following procedure can be used to measure the inhibitory effect of a compound on the HER2 receptor:

1) Implant, at various concentrations, MDA-MB-231 and MCF-7 cells transfected with HER-2 into the axillary mammary fat pads of female athymic mice;

2) Administer the compound; and

3) Measure the tumor growth at various time points.

The tumors can also be analyzed for the presence of a receptor such as HER2, by Western and immunohistochemical analyses. Using techniques known in the art, one skilled in the art can vary the above procedures, for example through the use of different treatment regimes.

(iv) Tumor Invasion Model

The following tumor invasion model has been developed and may be used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor.

(A) Procedure

8 week old nude mice (female) (Simonsen Inc.) were used as experimental animals. Implantation of tumor cells was performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10^7 tumor cells in a volume of 100 μl medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin was closed by using would clips. Animals were observed daily.

(B) Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases, to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurements of tumor size, grade of invasion, immunochemistry, and in situ hybridization).

The invention illustratively described herein may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

Those references not previously incorporated herein by reference, including both patent and non-patent

references, are expressly incorporated herein by reference for all purposes.

Other embodiments are encompassed by the following claims.

CLAIMS

What Is Claimed Is:

1. Method for modulating the activity of a protein kinase comprising the step of contacting said protein kinase with an indolinone compound demonstrated to have modulating capability in a bioassay corresponding to said protein kinase.

2. The method of claim 1, wherein said protein kinase is a serine kinase.

3. The method of claim 1, wherein said protein kinase is a threonine kinase.

4. The method of claim 1, wherein said protein kinase is a tyrosine kinase.

5. The method of claim 4, wherein said tyrosine kinase is a split kinase.

6. The method of claim 4, wherein said tyrosine kinase is a non-split kinase.

7. The method of claim 1, wherein said bioassay is a Flk bioassay.

8. The method of claim 1, wherein said bioassay is an EGF bioassay.

9. The method of claim 1, wherein said bioassay is a HER2 bioassay.

10. The method of claim 1, wherein said bioassay is a PCRB bioassay.

11. The method of claim 1, wherein said bioassay is a BIOKIN bioassay.

12. The method of claim 1, wherein said bioassay is a rafmak bioassay.

13. The method of claim 1, wherein said method is performed in vitro.

14. The method of claim 1, wherein said method is performed in vivo.

15. The method of claim 14, wherein said method at least partially alleviates or prevents one or more symptoms associated with a cell proliferation or cell differentiation disorder.

16. The method of claim 15, wherein said protein kinse is a PDGFR kinase and said disorder is selected from the group consisting of cancer, arterial

restenosis, fibrotic disease of the lung, kidney or liver, and wound scarring.

17. The method of claim 15, wherein said protein kinase is a FLk kinase and said disorder is selected from the group consisting of cancer, metastatic disease, rheumatoid arthritis, psoriasis, diabetic retinopathy, and age-related macular degeneration.

18. The method of claim 15, wherein said protein kinse is an EGFR or HER2 kinase and said disorder is selected from the group consisting of cancer and psoriasis.

19. The method of claim 15, wherein said protein kinse is a raf kinase and said disorder is selected from the group consisting of cancer, hyperplasia in arterial restinosis, transplant rejection, inflammation, and psoriasis.

20. A pharmaceutical composition comprising an compound of Table 1 and a physiologically acceptable carrier or diluent.

21. A method of preventing or treating an abnormal condition in an organism, where the abnormal condition is associated with an aberration in a signal transduction pathway characterized by an interaction

between a protein kinase and a natural binding partner, where the method comprises the following steps:

(a) administering a compound of Table 1 to an organism; and

(b) promoting or disrupting the abnormal interaction.

22. The method of claim 21, where the organism is a mammal.

23. The method of claim 21, where the protein kinase is a *FLK* protein kinase.

24. The method of claim 21, where the protein kinase is a platelet derived growth factor receptor protein kinase.